GENES ENCODING FOR THE HUMAN AND MURINE DEATH INDUCER-OBLITERATOR-1

insert B

The present invention relates to a novel DNA sequence that codes for expression of a human Death Inducer-Obliterator 1 (DIO-1)gene and the polypeptide derived from the DNA sequence. Expression vectors containing such sequences and host cells transformed with such expression vectors are also disclosed, as are methods for the expression of the novel DIO-1 polypeptide of the invention, and uses thereof.

Background

The binding of FasL or TNF to their specific receptors triggers oligomerization and activation of a series of events that results in apoptosis (Nagata, 1997). Dissection of the signal-transducing machinery for Fas-mediated apoptosis has revealed the presence of a set of molecules, FADD/Mort1, which is recruited by and associates with Fas following its activation. In a similar fashion, experiments with cells in which apoptosis was triggered by TNF-α show the existence of another protein, TRADD, which associates to TNFR1 and triggers cell death. TRADD binds to FADD through its death domain such that both stimuli, Fas and TNF-α activate the same downstream caspase pathway. TNF-α activates still another apoptosis pathway through the recruitment of RIP, a serine/threonine kinase that activates the apoptotic pathway by yet unknown mechanisms. More recently, it has been clearly established that STAT1 is required for TNF-α -triggered cell death (Kumar *et al.*, 1997; Hoey, 1997).

Three death factors, namely TNF, FasL and TRAIL (Pan *et al.*, 1997; Gura, 1997), as well as four death receptors, Fas, TNFR1, DR3/Wsl-1 and CAR 1 (Walczak *et al.*, 1997; Chinnaiyan *et al.*, 1996), have to date been shown to play a role in apoptosis triggering. Loss-of-function mutations in the Fas system illustrate the relevance of this death factor system in maintaining lymphocyte homeostasis. The binding of these factors to their specific receptors triggers a cascade of specific cysteil proteases, the caspases, which cleave various cellular components and lead to the morphological changes characteristic of apoptosis in cells and nuclei. The known signaling pathway initiates at the cell surface and operates in the cytoplasm, the main location of the caspases as well as their inhibitors (Vucic *et al.*, 1997; Irmler *et al.*, 1997; Ghayur *et al.*, 1997; Vaux, 1997; Chinnaiyan *et al.*, 1997). Very little is known

as to how these signals are transmitted to the nucleus. A caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) have recently been identified in the cytoplasmic fraction of a mouse lymphoma cell line. Caspase pathway activation by different stimuli cleaves ICAD, allowing CAD to enter the nucleus and degrade chromosomal DNA (Enari *et al.*, 1998; Sakahira *et al.*, 1998).

The invention

The invention relates to the DNA sequence, amino acid sequences and compounds and methods as defined in the claims.

It also relates to the use of the new gene as defined in the claims.

The new gene has been called DIO-1 gene by the inventors.

The terms "variants" and "alleles" mean that they are derived from the sequences given in the figures and have the same function as those.

For the purpose of the invention, gene means both genomic DNA, cDNA, and synthetic DNA.

The claimed nucleotide and amino acid sequences are new. They have been found to be useful for control of apoptosis and thereby useful not only for the treatment of diseases which are characterized in the alteration in cell death or by hyperproliferation, but also for the treatment of metabolic, proliferative or inflammatory conditions.

As examples cancer, autoimmune diseases, diabetes, rheumatoid arthritis, benign and malignant tumors and hyperproliferative skin disorders can be mentioned.

Figure legends

Figures 1 A, B, C and D and E. Nucleotide and predicted amino acid sequences of DIO-1. The bipartite NLS sequence is boxed and the zinc finger motifs are underlined.

Figure 1 E. Schematic representation of the predicted murine DIO-1 ORF. The starting and ending positions of the amino acids defining the motifs are numbered on top.

Figures 2. a - 2e. Northern blots and Western blot analysis, cell death and DIO-1 expression analysis.

Figures 3 a - 3c. DIO-1 expression pattern during murine limb development

Figures 4A- 4F. Overexpression of DIO-1 in chick limb.

Figure 5A - 5D. Expression pattern of several transcription factors in DIO-1-infected chick limb bud.

Detailed description of the invention

Methods

Cloning of DIO-1.

Differential display experiments were carried out using an RNAmap kit (GenHunter Corp.,) according to the manufacturer's specifications. Briefly, 200 ng of total cytoplasmic RNA (after DNase treatment with the MessageClean Kit; GenHunter) isolated from WOL-1 cells at 0, 2, 4 and 8 h after IL-7 withdrawal were reverse-transcribed with oligo(dT) primers ($T_{12}MN$) in the presence of Moloney murine leukemia virus reverse transcriptase. They were then amplified with several combinations of 5' decamer arbitrary primers and the $T_{12}MN$ used for RT in the presence of ^{35}S-dATP (1200 Ci/mmol). The amplified products were resolved in an 8 M urea, 6% polyacrylamide DNA sequencing gel and analyzed by autoradiography. Several bands of interest were isolated, reamplified, cloned in the pCR-Script SK(+) vector (Stratagene, La Jolla, CA) and further used for Northern analysis and sequencing. The full-length DIO-1 cDNA was obtained from WOL-1 cDNA by 5' RACE using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), with a 3' primer called L282 (5'-AGGTGTACCTTGTACAGCAGTGAAAC-3'). The resulting 2.6 Kbp band was excised from the gel and cloned in the TA-type vector pGEM-T (Promega, Madison, WI). The resulting clones were sequence-analyzed for orientation, and the oriented sense with respect to the T7 promoter was called DIO-1pGEM-T.

To confirm the ORF sequence obtained, a cDNA library from mouse brain cloned in λZAPII (Stratagene) was screened by probing with the RACE clone: the same probe was used to screen a human fetal kidney cDNA library (Clontech) from which the human DIO-1 homologue was cloned.

Cells and transfections.

WOL-1 cells were derived from the bone marrow of adult BALB/c mice. They are an untransformed, IL-7-dependent, stroma cell- independent pre-BI cell line, capable of reconstituting irradiated SCID mice. WOL-1 cells grow in Iscove's modified Dulbecco's medium (IMDM) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 1 mM sodium pyruvate, non-essential amino acids, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 10% fetal calf serum (FCS) and IL-7 (3% supernatant from a murine IL-7-producing cell line). A20, BAF/3, and FL5.12 cell

lines were maintained in RPMI 1640 with 10% FCS as described, and the FL5.12hBcl-2 stable cell line was grown in the presence of 1 mg/ml G418. MEF(10.1)Val5MycER cells were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) containing 10% FCS at 39°C. Where indicated, 1 μM E2 (17b-estradiol) was added to the medium to activate the MycER fusion protein after 24 h FCS starvation (Wagneer *et al.*, 1994). WOL-1, A20, BAF/3, and FL5.12 cell lines were cultured at 37°C, and all cell lines were kept in a humidified atmosphere with 5% CO_2.

Transient DNA transfection was performed by electroporation. For each transfection, 2 x 10^6 log phase cells were collected by centrifugation, resuspended in 200 μl of complete RPMI 1640 medium without FCS. After addition of 10 μg of plasmid DNA (1 mg/ml), samples were gently shaken and electroporated in a 0.4 cm electrode gap gene pulser cuvette at 960 μF and 320 V with a GenePulser apparatus (Bio-Rad, Hercules, CA). Samples were then diluted with 6 ml of the same medium supplemented with 10% FCS and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were analyzed for cell cycle staining by FACS at 48 h post-electroporation.

Northern blot analysis.

Total cytoplasmic RNA was prepared as described (Sambrook). RNA (10 μg) was Northern blotted using a ^{32}P-labeled DIO-1 riboprobe made by DIO-1pGEM-T digestion with *Bgl* II and *in vitro* transcribed from SP6 using the Riboprobe *in vitro* Transcription System (Promega). Hybridization was performed in 50% formamide at 65°C; washes were in 0.1X SSC + 0.1% SDS at 80°C. Blots were exposed on Kodak X-OMAT AR films at -80°C with two intensifying screens.

Antibodies and Western blotting.

A peptide was synthesized corresponding to amino acids 58-72 of murine DIO-1 with an additional N terminal cysteine (CSLRRSGRQPKRTERV); it was then coupled to maleimide-activated keyhole limpet hemocyanin and the purified conjugate injected into New Zealand White rabbits. Polyclonal antibody was affinity purified against the peptide coupled to a column. WOL-1 were IL-7 starved by washing four times in complete IMDM without FCS, then resuspended in the same volume of medium plus 10% FCS.

In situ hybridization and histology

Whole mount *in situ* hybridization was carried out as described (Wilkinson, 1993) with some minor modifications (Izpisúa Belmonte *et al.*, 1993). The DIO-1 digoxigenin probe was made by *Bgl*II digestion of the DIO-1pGEM-T and transcription from the SP6 promoter. The probe used for *Lhx-2* (700 bp) encompasses the homeobox and the second LIM domain. The remaining probes have been described elsewhere, and include *Msx-1* (Robert *et al.*, 1991), *Fgf-8* (Vogel *et al.*, 1995), *NF-κB* (Kanegae *et al.*, 1998). To visualize the cartilage, embryos were fixed in trichloroacetic acid after viral infection, stained with 0.1% alcian green and dehydrated/cleared in methyl salicylate.

Production of virus and injection protocols

Chicken embryos (either from MacIntyre Poultry, San Diego, CA, or SPAFAS, Norwich, CT) were infected with a virus containing the full length cDNA of DIO-1. Virus preparation and injections were as previously described (Morgan *et al.*, 1992). After injection, the embryos were returned to the incubator at 37°C and fixed at different time points either for *in situ* hybridization or for phenotypic analysis.

Example 1

To search for genes implicated in apoptosis, the differential display PCR technique was used (Liang and Pardee, 1992) with mRNA obtained from the WOL-1 pre-BI cell line as target. WOL-1 was derived from BALB/c adult bone marrow; it grows exponentially in the presence of IL-7 and undergoes apoptosis upon IL-7 withdrawal. Using a set of oligonucleotide primers, one specific for the polyadenylated tail and the other arbitrary in sequence, mRNA was amplified from cells in exponential growth or 2 h after IL-7 deprivation; this was followed by RNA reverse transcription and resolution on a denaturing sequencing gel. Of 82 positive bands, 10 were initially identified as undergoing either upregulation or downregulation during apoptotic death and were therefore considered candidates for further analysis. They were further amplified, sequenced and compared with known gene sequences using the NCBI BLAST program. Of these, one band (Death Inducer- Obliterator 1, DIO-1) revealed that the nucleotide sequence was a novel gene in that it showed no significant identity to any known gene or translated products in the data bases. Close inspection of the DIO-1 gene showed a stretch of nucleotides with homology to Zn finger domains, nuclear localization signals, and acidic transcriptional activating domains in the amino terminal domain. Northern blot analysis of mouse tissue using a labeled DIO-1 probe

identified a major mRNA species of 9.5 Kb (see below). The cDNA clone encoded an open reading frame of 614 amino acids (Fig. 1A). In the 3' untranslated region both TA tracts and a poly(A) tail were identified. DNA searches confirmed that this nucleotide sequence was that of a novel gene.

Nucleotide and predicted amino acid sequences of human and murine DIO-1 are shown in Figures 1A, B C and D respectively. The bipartite NLS sequence is boxed and the zinc finger motifs are underlined.

The DIO-1 protein does not belong to any typical family presently identified, and comprises an N-terminal domain, a central non-canonical Zn finger domain, and a C-terminus domain containing a K-rich region. See Figure 1F which is a schematic representation of the predicted DIO-1 ORF. The starting and ending positions of the amino acids defining the motifs are numbered on top.

Example 2

To further characterize DIO-1, its expression pattern was examined by Northern blot analysis using a DIO-1 probe of RNA samples isolated from several cell lines in exponential growth or undergoing apoptosis following triggering in different conditions. Northern blots containing 10 mg per lane of total cytoplasmic RNA from the indicated cell lines, treated with several apoptotic stimuli at different time points, were hybridized to the DIO-1 riboprobe. The blots were reprobed with an actin probe for normalization of the amounts loaded. Figure 2a shows that WOL-1 in exponential growth phase expresses low levels of DIO-1, which increase upon induction of apoptosis . Conclusion: DIO-1 is upregulated in cells deprived of IL-7, or treated with IFN-γ or with dexamethasone, but not in cells treated with etoposide, UV irradiation or in those undergoing Fas-mediated cell death

Figure 2b shows Western blot analysis of WOL-1 cells driven to apoptosis by IL-7 starvation. The cells were collected at different time points after removal of IL-7 from the culture medium; 5 x 10^5 cells were lysed with RIPA buffer and the total extract electrophoresed on an 8% PAGE-SDS gel, blotted and incubated with a affinity-purified polyclonal antibody against amino acids 58-72 that specifically recognizes DIO-1 (1:100 dilution in TBS-1% dry milk). Equivalence of protein loading was confirmed by Ponceau S staining. The position of the DIO-1 gene product is indicated in Figure 2b.

The upregulation of DIO-1 mRNA levels in cells undergoing apoptosis was thus confirmed in Western blot, using the polyclonal antibody anti-DIO-1. Figure 2b shows that in cell extracts derived from WOL-1 cells undergoing IL-7 deprivation-induced

apoptosis, but not after etoposide-induced cell death, a 67 kDa band is upregulated two hours after induction. In all cases, there is a correlation between the kinetics of cell death and the upregulation of the mRNA encoding DIO-1 or of the DIO-1 protein itself.

Example 3

The effect on cell death was initially examined following transfection of DIO-1 into several cell lines using a transient transfection assay.

The DIO-1 ORF was cloned into the pcDNA3 mammalian expression vector (Invitrogen, Inc., San Diego, CA). Both empty vector and the DIO-1 construct were transiently transfected by electroporation into A20 and BAF/3 cell lines. After 48 h expression, the cells were permeabilized and stained with propidium iodide, and cell cycle analyzed by FACS. Under the same conditions, both FL5.12 wild type and stably transfected hBcl-2 cells were transiently transfected.

In Figure 2c it is shown that transfection of a DIO-1 expression plasmid into BAF/3 cells results in a dramatic loss of cell viability at 48 h post-transfection, and cells displayed morphological alterations characteristic of apoptosis, becoming rounded, condensed and finally, dying. This effect was specific in that transfection of BAF/3 with an empty vector had no effect on cell survival. To verify these results, DIO-1 plasmids were transfected into A20 (Fig. 2c) or FL5.12 cells (Fig. 2d) In Figure 2d it is shown that hBcl-2 suppresses DIO-1-induced cell death in FL5.12 cells

Conclusion: Transient transfection with plasmids containing DIO-1 induced cell death in both cases, with kinetics similar to those observed for BAF/3. When DIO-1 was transfected in FL5.12 cells overexpressing human Bcl-2, cells were resistant to cell death, showing that Bcl-2 coexpression inhibits DIO-1 death-promoting activity. In MEF(10.1)Val5MycER cells, when plasmids encoding full-length DIO-1 were transfected, cells exhibited apoptotic morphology as assessed by 4'6'-diamidino-2-phenylindole (DAPI) staining (not shown). Furthermore, using the DIO-1-specific antibody, we find that DIO-1 is located in the cytoplasm of MEF(10.1)Val5MycER cells in exponential growth. When apoptosis is triggered by addition of E2 (17b-estradiol) (Wagneer *et al.*, 1994), DIO-1 is translocated to the nucleus in apoptotic cells (not shown). Extensive efforts to derive stable DIO-1 transfectants in these three cell lines were unsuccessful, suggesting the lethality of DIO-1 expression in these cells (not shown).

DIO-1 is thus differentially expressed under several apoptotic conditions and induces apoptosis when overexpressed.

Example 4

DIO-1 expression was analyzed in murine tissues by hybridization with the DIO-1 riboprobe of a mouse MTN Blot (Clontech). To determine DIO-1 transcript distribution, various tissues were analyzed in Northern blot. Two mRNA transcript bands corresponding to 9.5 and 5.4 kb were detected in most tissues tested, including thymus, spleen, heart, brain, lung, liver, skeletal muscle, kidney and testis. This expression pattern was confirmed with the anti-DIO-1 antibody in Western blot. DIO-1 expression was also upregulated *in vitro* in various cell lines, derived from different tissues, when undergoing apoptosis

The result is shown in Figure 2e. Molecular size markers are indicated on the left. Lanes: 1, heart, 2, brain, 3, spleen, 4, lung, 5, liver, 6, skeletal muscle, 7, kidney, 8, testis.

Example 5

The development of the vertebrate limb is an amenable system for the study of signaling pathways leading to tissue patterning, proliferation and cell death (Izpisúa-Belmonte *et al.*, 1993; Schwabe *et al.*, 1998). Limbs originate as a consequence of the differential growth of cells from the lateral plate mesoderm at specific axial levels (Summerbell *et al.*, 1973). At the tip of the limb primordium, a morphologically homogenous and rapidly proliferating group of mesenchymal cells, called the progress zone, induces the overlying ectoderm to differentiate and form a specialized structure termed the apical ectodermal ridge (AER). Subsequent limb outgrowth and maintenance of the AER requires reciprocal signaling between the ridge and the underlying mesodermal cells of the progress zone (Todt and Fallon, 1984; Morgan *et al.*, 1992; Wilkinson, 1993; Vogel *et al.*, 1996). A process that involves programmed death of mesenchymal cells is also required, and a specific gene, BMP-4, has been implicated in this process. DIO-1 expression during mouse fetal development was tested and it was found that it is expressed during limb development.

DIO-1 expression pattern during murine limb development by whole mount *in situ* hybridization is shown in Figure 3a-c. BALB/c embryos from days 10.5 (a), 11.5 (b) and 12.5 (c) were hybridized to DIO-1 digoxigenin probe, showing expression in the postero-distal zone (a) of the limb. In (b), the pattern is clearly distal, with remarkable indentation in the nascent interdigitating spaces, while in (c), the expression is in the

interdigitating spaces, with no DIO-1 in the apical zone of the fingers, exhibiting another clear pattern in two areas of the antero-posterior axis.

As shown in Figure 3a-c, DIO-1 is highly expressed at gestation day 12.5 in the interdigitating membranes, where programmed cell death is known to occur.

Example 6

Cell growth, cell differentiation and cell death are intimately linked during the development of the vertebrate embryo; the developing limb is perhaps one of the best model systems in which to study this process (for a review, see Schwabe *et al.*, 1998). The AER is a pseudostratified epithelium located at the distal part of the developing limb bud shown to be required for limb outgrowth (Summerbell *et al.*, 1973; Todt and Fallon, 1984). Subsequent to the alteration in AER formation, limb outgrowth is arrested. To better understand the role of DIO-1, retroviral technology was used to misexpress it in the chick limb. A retroviral vector containing the *RCAS-Dio-1* construct, the DIO-1-ORF, was injected into the limb primordia of chick embryos at stages 8-12. At 60-72 h after injection, infected limb buds failed to develop a normal AER. Embryos were examined at different stages following infection.

The result is shown in Figures 4A-F in which A is whole mount preparation showing the leg of a wild type embryo. B is Alcian green staining of the same limb to visualize the normal cartilage pattern. C shows an infected embryo 6 days after injection showing extensive truncation of the distal elements of the leg. D shows same embryo after cartilage staining. Note the complete absence of elements distal to the tibia-fibula joint. E and F show the whole mount and cartilage staining of an embryo 8 days after infection with the *RCAS-Dio-1* construct.

In most cases, the truncations occur in the most distal elements (showing an absence of digits, carpals and metacarpals (Figure 4C and 4D). In others, reduction in size and malformations of the tibia and fibula are observed (Figure 4E and 4F and data not shown).

The infected limb is distorted and reduced in size, exhibiting an absence, reduction or malformation of phalanges, tarsals and metatarsals (dot). In a few cases, the fibula was reduced in size (asterisk).

Conclusion: Overexpression of DIO-1 inhibits chick limb outgrowth.

Example 7

Since misexpression of DIO-1 can perturb AER formation, it could be expected that it is preceded by changes in gene expression, both in the ectoderm and in the underlying limb bud mesoderm. *In situ* hybridization of embryos were infected with the *RCAS-*

Dio-1 construct using riboprobes for mesodermal genes involved in limb outgrowth such as *Msx-1*, *Lhx*-2 , and *NF-κB* (Kanegae *et al.*, 1998). *Msx-1* *Lhx*-2 , and *NF-κB* (show downregulation in their transcript levels. see Figure 5A, 5C and 5D, respectively. Furthermore, transcripts for ectodermal genes involved in limb outgrowth, such as *Fgf-8* , are also absent or downregulated. see Figure 5B). Note the reduced size of the infected limb buds (left limb buds in all cases). Transcripts for *Msx-1* (A), *Fgf-8* (B), *Lhx-2* (C) and *NF-κB*(D) are strongly downregulated (arrows) in the injected limb buds (compare with the normal expression pattern in the uninjected limb bud, right limb bud in all cases). Misexpression of the *RCAS-Dio-1* construct thus leads to arrest in limb outgrowth that is preceded by changes in the expression of genes involved in outgrowth of the limb. It is not known whether the misexpression of DIO-1 is directly responsible for the downregulation of ectodermal gene markers (i.e., *Fgf-8*) or if this is a consequence of the previously altered mesodermal gene expression. The combination of these results indicates that DIO-1 may regulate cell death and proliferation during limb development.

Conclusion: DIO-1 overexpression alters gene expression in the developing chick limb bud.

Discussion

Cell growth, cell differentiation and cell death signals are regulated through triggering of specific receptors, which leads to the activation of specific mediators, giving rise in turn to gene transcription activation and/or postranslational modification. Nonetheless, the mechanism through which this activation takes places has not yet been identified. Much work has been done on the mechanism activated by Fas and TNFR ligation in the triggering of apoptosis, but we know very little of the mechanism implicated in transcriptional regulation during the apoptotic process. Few of the molecules presently associated with apoptosis regulation are transcriptional regulators, among which p53 (Wagneer *et al.*, 1994), Nur77 (Chong *et al.*, 1997), the glucocorticoid receptor, STAT1 (Kumar *et al.*, 1997) and *NF-κB* (Kanegae *et al.*, 1998) are probably the only ones identified so far. Neither Fas- nor TNF-α-mediated apoptosis require gene transcription for induction; it may nonetheless play a role in these cases. We have recently shown that Nur77 is upregulated during Fas-mediated apoptosis, and that constitutive expression of Nur77 renders cells more susceptible to Fas-induced death. On the contrary, nuclear translocation of NF-κB prevents Fas- and TNF-α -triggered cell death, and constitutive expression of IκB favors cell death.

The structure and features of DIO-1 makes this gene useful for control of apoptosis.

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